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Abstract: PURPOSE: Chronic tearing of tendons is associated with molecular and structural alterations causing biomechanical changes, which compromise musculotendinous function and become limiting factors for tendon repair. This study investigated the histological response of chronically retracted sheep rotator cuff tendons to mechanical and pharmacological stimulation in view of tendon repair. METHODS: Sixteen weeks after experimental release of the infraspinatus tendon in 20 sheep, the retracted musculotendinous unit was subjected to continuous traction either with [anabolic steroids (nandrolone) group/insulin-like growth factor (IGF) group] or without (control group) additional pharmacological treatment during 6 weeks. A new degeneration score for tendinous tissues (DSTT), based on established knowledge on histological changes associated with tendon degeneration, was used for histological analysis at the time of tendon release, at the beginning of continuous re-lengthening and at repair in all animals. RESULTS: The DSTT score (inter-observer correlation: r = 0.83), quantifiably representing tendon degeneration, improved from 15.5 (SD 1.3) points before to 9.8 (SD 3.8) points after re-lengthening. It improved in a qualitatively and quantitatively similar fashion if pharmacological stimulation was added. The nandrolone group improved from 13.7 (SD 1.6) to 9.8 (SD 1.9) and the IGF group from 13.3 (SD 3.6) to 8.8 (SD 1.8) points. CONCLUSION: Mechanical stimulation significantly reduced tissue degeneration. However, the addition of a pharmacological stimulation with anabolic steroids or IGF had neither a measurable positive nor negative effect on the degenerative process. Therefore, this investigation does neither support the additional pharmacological use of the anabolic steroid nandrolone or of IGF decanoate for restoration of tendon degeneration, nor otherwise provide evidence for additional tendon damage, if those substances are used to alter the muscular metabolism.

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SHOULDER

Tendon response to pharmaco-mechanical stimulation of the chronically retracted rotator cuff in sheep

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Methods Sixteen weeks after experimental release of the infraspinatus tendon in 20 sheep, the retracted musculotendinous unit was subjected to continuous traction either with [anabolic steroids (nandrolone) group/insulin-like growth factor (IGF) group] or without (control group) additional pharmacological treatment during 6 weeks. A new degeneration score for tendinous tissues (DSTT), based on established knowledge on histological changes associated with tendon degeneration, was used for histological analysis at the time of tendon release, at the beginning of continuous re-lengthening and at repair in all animals.
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Keywords Tendon degeneration · Rotator cuff · Sheep rotator cuff model · Pharmaco-mechanical stimulation · Tendon re-lengthening · Anabolic steroids · Nandrolone · IGF

Introduction

Musculotendinous retraction is recognized as a pathophysiological consequence of chronic tendon tearing and as the major limitation for successful operative tendon to bone repair [18]. In addition to degenerative muscular changes, also the tendon deteriorates showing mucoid degeneration, fatty infiltration and calcification as well as alterations in collagen architecture and fibre alignment [5, 7, 15, 16]. Continuous traction and re-lengthening of the chronically retracted musculotendinous unit has previously been shown to not only stop fatty infiltration of the muscle [9] but also

Karl Wieser and Mazda Farshad have equal contribution to this study.

This study was performed at the Balgrist University Hospital, University of Zurich, Zurich, Switzerland; and the Vetsuisse Faculty, University of Zurich, Zurich, Switzerland.

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to arrest and partially restore degenerative tendinous changes with a reduction in over-crimping of the collagen fibres and to partially restore collagen fibril morphology and assembly [7]. To prevent or to restore muscle degeneration, the use of anabolic steroids and/or insulin-like growth factor (IGF) is currently under investigation for potential adjuncts in the treatment of rotator cuff tears. However, potential influence of collagen expression, biosynthesis and turnover [4, 12, 14] in ligaments and tendons raises concern of progressive tendinous degeneration.

The present series of investigations in a rotator cuff sheep model [8, 9] was therefore designed to test the hypothesis that the addition of either anabolic steroids or IGF leads to better musculotendinous recovery after repair of retracted rotator cuff tears than continuous musculotendinous traction alone (referred to as mechanical stimulation). The effects on muscle have been reported elsewhere [11]. However, as tendon degeneration is believed to be an important clinical factor if a rotator cuff repair is considered, it is the purpose of this study to report the potential histological changes induced by anabolic steroids or IGF in experimentally degenerating rotator cuff tendons. To be able to reproducibly assess those histological changes, we modified an existing semi-quantitative, histological degeneration score for tendinous tissue (DSTT) incorporating recent knowledge on characteristic alterations of degenerated tendons.

Materials and methods

Surgical technique and experimental protocol

A detailed description of the surgical technique and time protocol has previously been published [11]. The surgical procedure and the tendon-specific data acquisition and analysis can be summarized as follows:

Tendon release (biopsy A)

In 20 Swiss female alpine sheep [age 23 (±2) months; weight 55 (±5) kg)], the right infraspinatus (ISP) tendon was released using an osteotomy of the greater tuberosity, immediately after initial biopsy of the ISP tendon (*biopsy A*). The biopsy site was marked with non-resorbable USP no. 4–0 monofilament suture, and the tendon–bone chip complex was wrapped in a silicone tube to prevent scarring and was allowed to retract for a total of 4 months.

Implantation of traction device (biopsy B), re-lengthening and repair

Following this period, a second biopsy of the tendon (biopsy B) was performed next to the first biopsy site, which was again marked with a second suture. A device, allowing continuous lengthening (1 mm/day) of the musculotendinous unit by external manipulation of the lever arm, was then implanted on the scapular spine of all sheep (Fig. 1). The tendon–bone chip complex was grasped with 2 USP No. 2 FiberWire sutures (Arthrex, Naples, Florida), which were connected to the traction device. After the re-lengthening period of 6 weeks, during which the sheep could ambulate freely, the traction device was removed and the rotator cuff was repaired by re-attaching the bone chip to its original site or as near to this site as possible with attachment of the remaining sutures to a 3.5-mm cortical bone screw with a washer. For the first 6 weeks after this repair, a suspension belt and a ball, attached to the sheep's hooves, were used to prevent the sheep from stressing the repaired tendon. Subsequently, a full weight-bearing period of 8 weeks was allowed before killing.

Killing

All animals were killed 14 weeks after repair, and a third tendon biopsy (*biopsy Cop*) was taken. At this time, a biopsy was also taken on the intact contralateral control side (*biopsy Cco*).

Group distribution and pharmacological treatment

The 20 sheep were randomized into three different intervention groups before the first surgical procedure.

In the first group of 7 sheep (*control group*), no pharmacological stimulation was used.

In a second group (*nandrolone group*, $n = 7$), 3 ml of nandrolone decanoate (50 mg/ml) was injected into the infraspinatus muscle immediately adjacent to the scapular spine before skin closure. An additional 3 ml of nandrolone decanoate (50 mg/ml) was injected every two weeks for

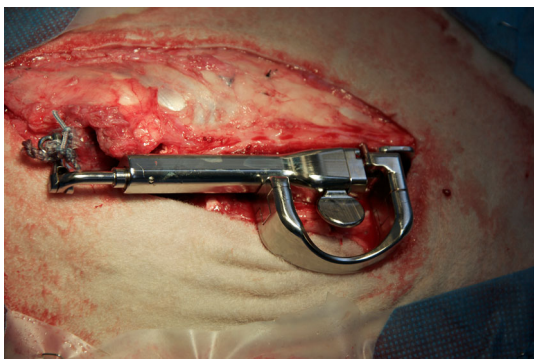

Fig. 1 Intra-operative picture of the tendon–bone chip complex, which is connect with 2 USP No. 2 FiberWire sutures (Arthrex, Naples, Florida) to the implanted traction device. This traction device allows non-invasive control of re-lengthening of the central rod by 1 mm/day

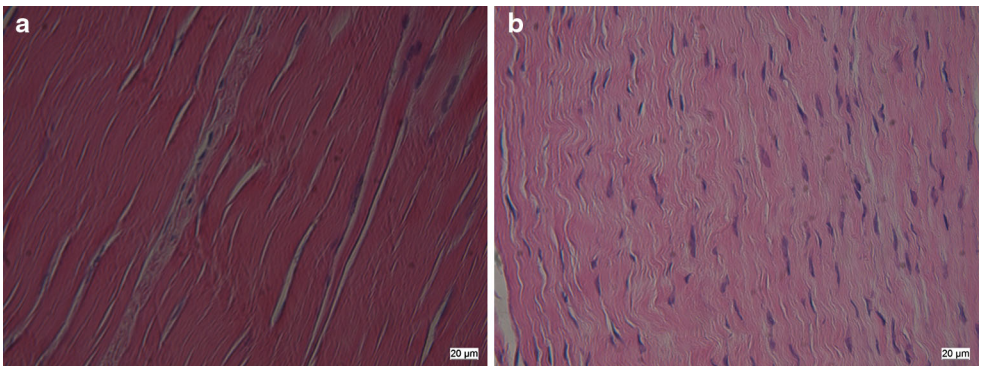

Fig. 2 **a**, **b** H&E staining; 20 × magnification; **a** a field of view of a healthy tendon with less than 50 tenocytes and small and fusiform (spindle-shaped) cell nuclei (DSTT 0 points). b A severely degenerated tendon with more than 150 tenocytes and the majority of cell nuclei rounded and bulky (DSTT 3 points)

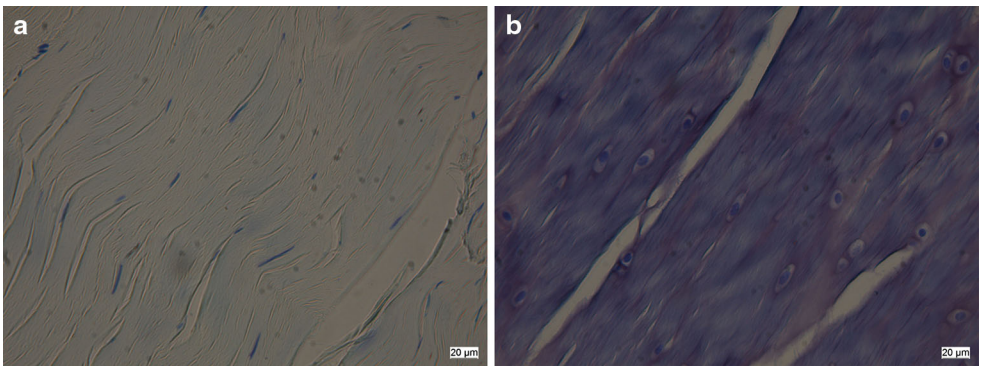

Fig. 3 **a**, **b** Toluidine blue staining; 20× magnification; **a** week toluidine staining (score 0 points). **b** Intensively toluidine reaction reflecting an increased amount of proteoglycan (score 3 points)

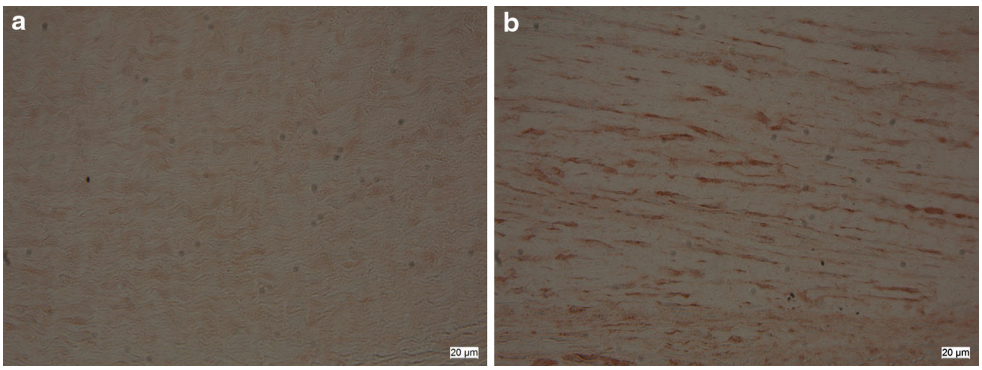

Fig. 4 **a**, **b** Decorin staining; 20× magnification; **a** weak Decorin staining (score 0 points) and **b** pronounced Decorin staining (score 2 points)

the period of re-lengthening into the scapular spine-sided part of the ISP muscle belly.

In a third group (*IGF group*, $n = 6$), 267 µg IGF (3.07 µg IGF-I loaded per mg PLGA microsphere with a loading efficacy of 35 %) was applied to the ISP muscle belly adjacent to the scapular spine before skin closure. In animals treated with IGF, no additional injection was performed, as the substance was released continuously by the microspheres during the period of re-lengthening.

In six animals (three of the control and three of the nandrolone group), the attempt at repair failed at some point during the experiment and the respective specimens were excluded from this study. Two further animals, one of the control group and one of the nandrolone group could not be

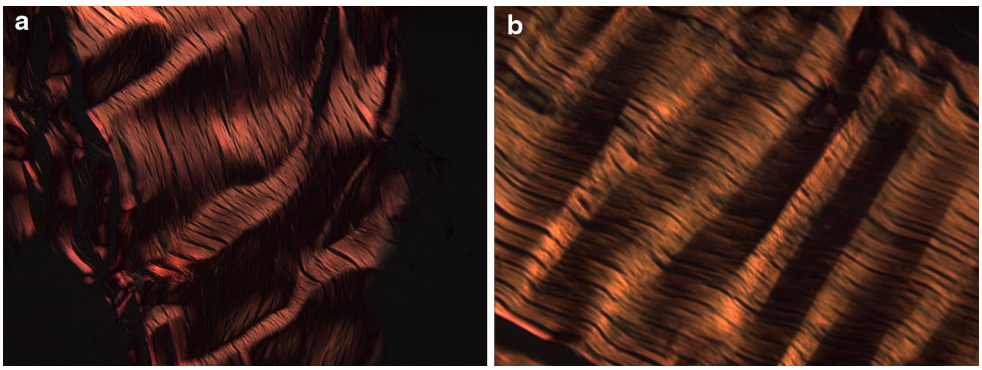

Fig. 5 **a**, **b** H&E staining; 20x magnification; polarized microscope; The figures show the gradual deterioration with **a** initial wave length of 150–200 µm (score 0 points) and **b** 50–100 µm (score 2 points), indicating advanced collagen fibre crimping

included into the histological analysis; the histological analysis of the tendon of one sheep showed massive tendon degeneration already present at the time point of tendon release. In the other animal, biopsies were avoided due to very marked fragility of the tendon at the time of implantation of the device in order to avoid iatrogenic tendon rupture.

Radiographic assessment

Computed tomography (CT) (Somatom AR.T; Siemens Medical Solutions, Erlangen, Germany) was performed at the time of each surgical intervention and at intervals of 2 weeks during the re-lengthening phase. Under general anaesthesia, the sheep were positioned in a standard lateral decubitus position on the uninvolved side. Slices were 2-mm thick, with an inter-slice gap of 5 mm. Musculotendinous retraction was measured as the distance of the bone chip to its original insertion side.

Histological processing

Tendinous specimens were embedded in 4 % formalin and processed with haematoxylin and eosin (H&E), toluidine blue and decorin. A haematoxylin solution modified according to Gill II (Merck, Darmstadt) and a Richard-Allan Scientific Eosin-Y w/Phyloxine (Distributor Microm International GmbH, Germany) with a Medite Tissue Stainer COT 20 (Burgdorf, Germany) was used for H&E staining. Unna's method for mast cells (polychrome methylene blue with differentiation in glycerine–ether) was used for toluidine blue sections. For decorin staining, tissue sections were first deparaffinized, rehydrated and counterstained with haematoxylin. Endogenous peroxidase was inhibited with 3 % H_2O_2 and a protein blocker (DAKO X0909) was used for another 10 min. The samples were then incubated after treatment with hyaluronidase (Sigma H3506330 U/mg) using Rabbit Ant Decorin LF 113 (Mouse, Larry W. Fisher, NIDCR, Bethesda, Maryland). Afterwards the En Vision ant rabbit (DAKO K4003) method was used and AEC (Aminoethyl Carbozole Substrate Kit, Invitrogen AG, 00-2007) was used as chromogen.

Degeneration score for tendinous tissues (DSTT)

Histological analyses were performed by two independent and blinded (group affiliation and time of biopsy) observers, using a scoring system adopted from studies assessing different tendinopathies [1, 15, 17, 22, 25]. The items included were (1) cell count (2) morphology of tenocytes (Fig. 2a, b), (3) (increased) vascularity, (4) development of chondroid metaplasia, (5) intensity of toluidine blue (Fig. 3a, b) and (6) decorin staining (Fig. 4a, b) (7) wavelength and (8) alignment of collagen fibres (Fig. 5a, b). Three representative fields of view were analysed using a Leica DMR microscope equipped with a 20-fold magnification objective or a polarization optics (used to visualize collagen fibre crimping). Each item was evaluated using a defined quantitative 4-grade system with higher scoring points indicating histological findings consistent with increased tendinous degeneration (0 indicating normal appearance, 1 indicating mild abnormality, 2 indicating moderately abnormal appearance and 3 indicating markedly abnormal appearance). The total score could thus vary between 0 (normal tendon) and 24 (most abnormal appearance detectable). The detailed scoring system is depicted in Table 1.

All animal experiments were performed according to Swiss laws of animal welfare and were authorized through the Animal Welfare Committee of the University of Zurich (authorization 89/2009).

Statistical analysis

After consultation with a biomedical statistician, descriptive statistical methods were employed to report the data with mean and standard deviation (SD). The overall inter-reader correlation was assessed treating values as continuous data, and Spearman's r was used to quantify the correlation.

Table 1 DSTT based on histological analysis

Cell count/field of view	
0	0–50 tenocytes cell nuclei
1	50–100 tenocytes cell nuclei
2	100–150 tenocytes cell nuclei
3	>150 tenocytes cell nuclei
Morphology (shape) of cell nuclei	
0	Majority of tenocytes cell nuclei are small and fusiform (spindle-shaped)
1	Majority of tenocytes cell nuclei are slightly broadened but still fusiform (spindle-shaped)
2	Majority of tenocytes cell nuclei are broadened and no longer fusiform (spindle-shaped)
3	Majority of tenocytes cell nuclei are rounded and bulky
Vascularity/fields of view	
0	No vessel
1	1–3 vessels
2	4–6 vessels
3	>6 vessels
Chondroid metaplasia/3 representative fields of views	
0	No sign of chondroid metaplasia in all 3 fields of view
1	Chondroid metaplasia in 1 field of view
2	Chondroid metaplasia in 2 fields of view
3	Chondroid metaplasia in all 3 fields of view
Toluidine stain	
0–3	Intensity of staining
Decorin stain	
0–3	Intensity of staining
Crimping of collagen fibres: wavelength (10 collagen fibre waves in 2 fields of views)	
0	150–200 μm
1	100–149 μm
2	50–99 μm
3	<50 μm
Crimping of collagen fibres: alignment of collagen fibres in 2 fields of views	
0	75–100 % of collagen fibres run parallel
1	50–75 % of collagen fibres run parallel
2	25–50 % of collagen fibres run parallel
3	0–25 % of collagen fibres run parallel
Total degeneration score	0–24

Results

Musculotendinous retraction of the animals included in this study, measured on CT scans, was 4.2 (SD 1.8) cm in the control group, 3.8 (SD 1.1) cm in the nandrolone group and 4.9 (SD 0.8) cm in the IGF group.

Table 2 Inter-observer correlation of histological analysis

	Inter-observer correlation (Spearman's *r*)
Cell count	0.86
Cell morphology	0.73
Vascularity	0.78
Chondroid metaplasia	0.75
Toluidine blue	0.59
Decorin	0.82
Fibre wavelength	0.84
Fibre alignment	0.85
Overall	0.83

The DSTT revealed a satisfactory inter-observer correlation of $r = 0.83$ and was considered as a reproducible scoring system for further group comparison. The correlation coefficients of each subgroup of the score, ranging from 0.59 to 0.86, are depicted in Table 2.

Tendon release (biopsy A)

At the time point of tendon release, the mean DSTT varied slightly between 5.5 (SD 0.9) in the control group, 5.3 (SD 1.2) in the nandrolone group and 5.2 (SD 1.6) in the IGF group. At this time point, all groups had a tenocyte cell count between 0–50 cells and less than three vessels/field of view with a wavelength of 100–200 μm and 50–100 % parallelism of the collagen fibres.

Implantation of traction device (biopsy B)

After 16 weeks of retraction, the DSTT increased to 15.5 (SD 1.3) in the control group, 13.7 (SD 1.6) in the nandrolone group and 13.3 (SD 3.6) in the IGF group. Cell count and cell morphology as well as vascularity, crimping and alignment of fibres showed the highest signs of degeneration (wavelength <50 μm; 0–50 % of collagen fibre parallelism) after this period of chronic musculotendinous retraction.

Killing (*biopsy C*)

At euthanasia, the operated shoulders (*biopsy Cop*) had a DSTT of 9.8 (SD 3.8) in the control group, 9.8 (SD 1.9) in the nandrolone group and 8.8 (SD 1.8) in the IGF group. Whereas the contralateral shoulders (*biopsy Cco*) showed values of 3.8 (SD 1.3) in the control group, 3.5 (SD 0.5) in the nandrolone group and 4.5 (SD 1.8) in the IGF group, respectively (Fig. 6). While wavelength, fibre alignment and cell morphology improved to almost normal values, the number of cells remained high after re-lengthening and reinsertion (Fig. 7).

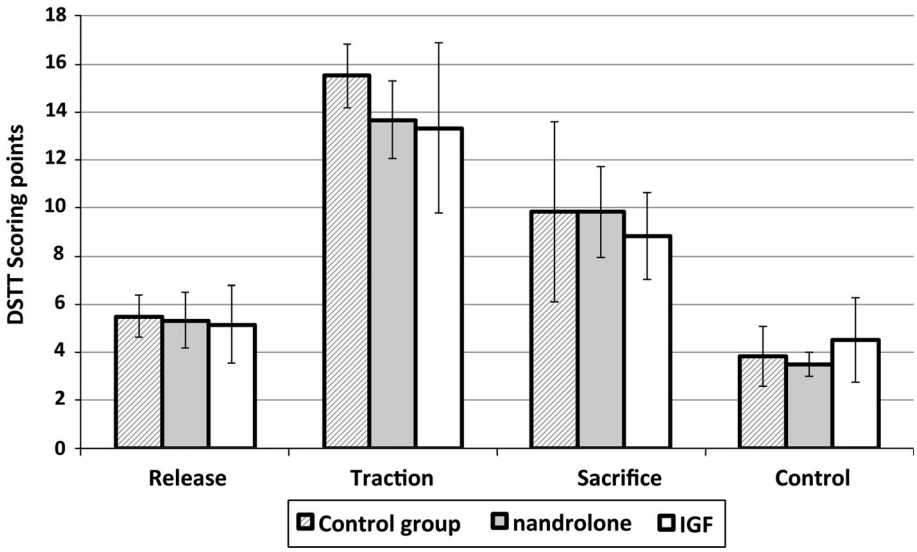

Fig. 6 At the time point of tendon release (*biopsy A*), the mean DSTT of the three groups varied slightly between 5.2 and 5.5. After 16 weeks, at the time point of implantation of the traction device (*biopsy B*), the DSTT increased to 15.5 (14–16) in the control group, 13.7 (12.5–15.5) in the nandrolone group and 13.3 (7.5–18.5) in the IGF group. At euthanasia, the DSTT of the operated shoulders (*biopsy Cop*) varied between 8.8 and 9.8, whereas the contralateral shoulders (*biopsy Cco*) showed values between 3.5 and 4.5, respectively

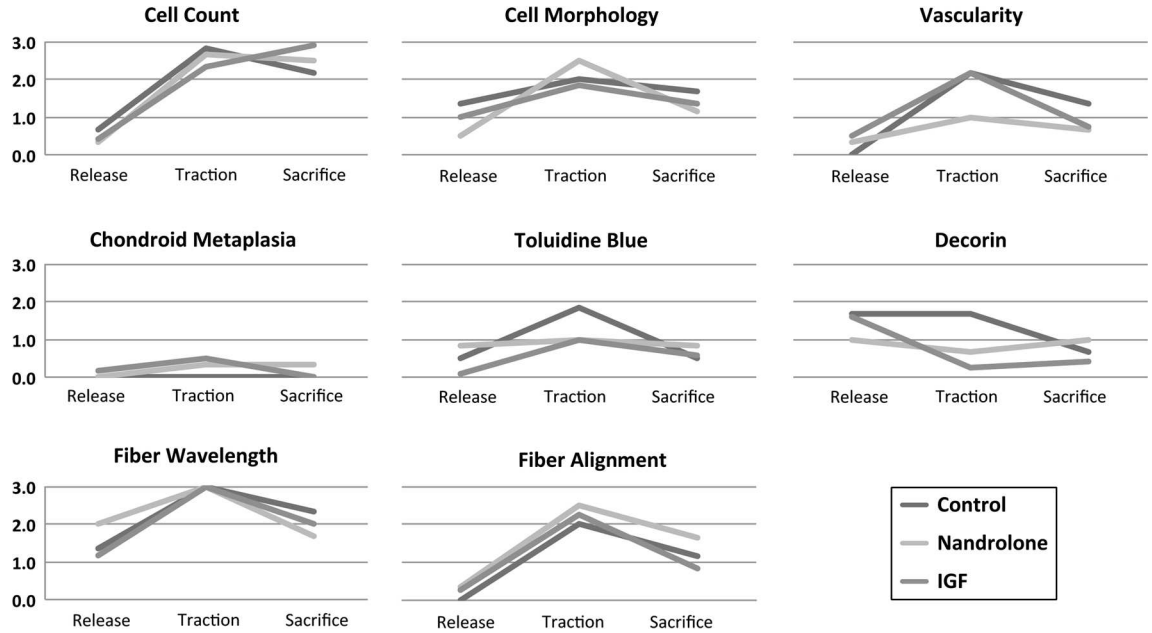

Fig. 7 Development of subcriteria of the DSTT in the control, the nandrolone and IGF group at the time points of tendon release (*biopsy A*), after 16 weeks (implantation of the traction device) (*biopsy B*) and at the time point of killing (*biopsy Cop*)

Overall neither the subgroup values nor the results of the total DSTT showed a clear tendency or clear differences between the groups with or without additional pharmacological treatment.

Discussion

The most important finding of this investigation was that continuous re-lengthening of the chronically retracted rotator cuff partially restores degenerative tendon alterations; however, additional pharmacological stimulation using the growth hormone IGF decanoate or the anabolic steroid nandrolone decanoate seems not have either a substantially positive or a negative effect on histological recovery of degenerated tendon tissue in the infraspinatus of the sheep. Despite confirmation of previous findings suggesting partial reversal of chronically retracted tendon degeneration with continuous musculotendinous traction [7], our hypothesis that the effects of mechanical simulation on the tendon are substantially influenced by the addition of a pharmacological stimulation has therefore to be rejected.

For best potential effect on the muscle, the chosen anabolic substances were nandrolone decanoate as a potent

anabolic steroid and IGF [11]. While the former was not expected to positively influence tendon degeneration, the latter was. Due to the fact that anabolic steroids are potent muscle growth stimulators and have the potential of preventing fatty infiltration of the muscle [10], they are of enormous interest in musculoskeletal research. In rodents, high doses of anabolic steroids seem to change collagen biosynthesis [12] leading to dysplastic collagen fibrils [19–21] with altered crimp pattern and angle [24]. This observation could neither be confirmed in the here employed sheep model nor by investigations of Evans and colleagues, who did not find ultrastructural collagen changes in ruptured distal biceps tendons of anabolic steroid users [6]. Tendon to bone healing, which is indispensable for successful rotator cuff repair, may be another issue of concern. Papaspiliopoulos et al. [23] showed decreased strength of the tendon to bone repair as well as fibroblastic reaction and inflammation in rabbits who received anabolic steroids. As due to the severe retraction, no direct tendon to bone healing could be achieved in the present model, this aspect could not be assessed.

Insulin-like growth factor, however, which has also been associated with muscle growth, and which is up-regulated in acute and chronic tendon healing [2, 3, 13], was expected to positively influence tendon tissue due to an anabolic potential for connective tissue by stimulation of collagen expression and synthesis [4] and induction of a higher collagen turnover in ligaments and tendons [14]. The fact that we did not find relevant differences between the groups, neither on the operated nor on the intact contralateral control side, is unexplained: suboptimal timing or dosage delivery method of IGF could be a factor, however, we must assume that with the high doses applied over a period of 6 weeks, if present, tendencies in tissue reaction should be discernable as the developed scoring system was designed to find even small differences in a systematic matter.

This new scoring system for histological assessment of tendon degeneration, which incorporates recent knowledge on characteristic alterations of degenerated tendon, was found to be reproducible with satisfying inter-rater correlation. However, we recognized that healthy tendon from young sheep showed a score of 2–6 out of 24 points, which was interpreted as the experimental consequence of mild fibre tension loss after biopsy, leading to reduced wavelength, which would formally present mild tendon degeneration in the definition of the score. Furthermore, despite good overall inter-observer correlation, the agreement of intensity of toluidine blue staining has to be considered as dissatisfying. Therefore, future studies have to evaluate whether minor adaptions of the scoring systems' cut-off values would be necessary to further validate this score and increase its experimental and clinical impact.

Despite observation of severe tendon degeneration after 16 weeks of musculotendinous retraction, we did not observe relevant chondroid metaplasia: this is in contrast to the literature reporting histological changes in chronic rotator cuff tendon tears [16]. The mechanism of development of cartilage-like changes like chondroid metaplasia is believed to be the consequence of shear and compressive instead of tensile forces. This is well documented in subacromial impingement in man; it seems, however, not to occur in our rotator cuff sheep model, in which a healthy tendon is released without any impingement during tearing and the retraction period. This discrepancy might be a limitation of the tear model. It cannot be fully excluded that the silicone tube around the bone chip and the most distal tendon did have an effect on the tendon. However, due to sheeps' tendency to massive scarring, performing this investigation without a silicon tube would make it impossible to obtain the necessary tendinous retraction. Although the usage of such a protective sleeve, preparation of the retracted tendon had to be performed very cautiously and we had to refrain from performing a biopsy in order to avoid iatrogenic rupture of one markedly fragile tendon. The sample size is another limitation of this study. The number of included animals allowed excluding a substantial influence of pharmacologic stimulation. Conversely, it does not, however, allow to exclude a minor, most likely clinically irrelevant influence.

Conclusion

This investigation does neither support the additional pharmacological use of the anabolic steroid nandrolone or of IGF decanoate for restoration of tendon degeneration, nor otherwise provide evidence for additional tendon damage, if those substances are used to alter the muscular metabolism.

Conflict of interest The authors have no potential conflict of interest.

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